THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 1, Issue of January 7, pp. 47–55, 2000

Printed in U.S.A.

Regulation of Rat Cytochrome P450C24 (*CYP24*) Gene Expression

EVIDENCE FOR FUNCTIONAL COOPERATION OF Ras-ACTIVATED Ets TRANSCRIPTION FACTORS WITH THE VITAMIN D RECEPTOR IN 1,25-DIHYDROXYVITAMIN D_3-MEDIATED INDUCTION*

(Received for publication, June 3, 1999, and in revised form, September 20, 1999)

Prem P. Dwivedi‡, John L. Omdahl§¶, Ismail Kola‖, David A. Hume, and Brian K. May‡**

*From the ‡Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5005, Australia, the §Department of Biochemistry and Molecular Biology, University of New Mexico, Albuquerque, New Mexico 87131, the ‖Molecular Genetics and Development Group, Institute of Reproduction and Development, Monash University, Melbourne, Victoria 3168, Australia, and the **Center for Molecular and Cellular Biology, University of Queensland, St. Lucia, Queensland 4072, Australia*

Transcription of the rat *CYP24* gene is induced by 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) through two vitamin D response elements (VDREs). A functional Ras-dependent Ets-binding site (EBS) was located downstream from the proximal VDRE and was critical to 1,25$(OH)_2D_3$-mediated induction. Cotransfection of Ets-1 and Ets-2 stimulated induction, which was lost when the EBS was mutated. Multiple nuclear-protein complexes from COS-1 cells bound to the EBS in which three complexes were immunologically related to Ets-1. Transcriptional synergy was observed between the proximal VDRE and adjacent EBS as was the attendant formation of a ternary complex between vitamin D receptor- retinoid X receptor (VDR·RXR) and Ets-1. In the absence of 1,25-$(OH)_2D_3$ or in the presence of an inactive proximal VDRE, the EBS failed to respond to exogenous Ets-1. However, Ets-1 increased basal expression when cotransfected with a mutant VDR. The inductive action of 1,25-$(OH)_2D_3$ was substantially increased by Ras, which was ablated by mutagenesis of the EBS or by expression of a mutated Ets-1 protein (T38A). EBS contribution to hormone induction was prevented by manumycin A, an inhibitor of Ras farnesylation. A fundamental role was established for transcriptional cooperation between Ras-activated Ets proteins and the VDR·RXR complex in mediating 1,25-$(OH)_2D_3$ action on the *CYP24* promoter.

The hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$[1] or calcitriol) is a pleiotropic secosteroid that directs many biochemical and cellular functions associated with calcium homeostasis, cellular proliferation and differentiation, and the immune response (1–4). The transcriptional regulation of target genes by 1,25-$(OH)_2D_3$ is mediated by the vitamin D receptor (VDR), a member of the ligand-dependent nuclear receptor superfamily (5–9). The liganded VDR in association with its partner retinoid X receptor (RXR) (a member of the nuclear receptor superfamily) binds to specific control elements referred to as vitamin D response elements (VDREs) that are located in the promoter of target genes (8–9). Current evidence suggests the VDR·RXR heterodimeric complex binds to the VDRE in the unliganded state (3, 10) and is subsequently activated following ligand binding. Transactivation of the target gene is achieved through an intermediary coactivator complex that functions to link the VDR·RXR receptor complex to the RNA polymerase II holoenzyme (10–12).

The level of expression of 1,25-$(OH)_2D_3$-responsive genes in tissues is dependent on the presence of VDR and the concentration of intracellular 1,25-$(OH)_2D_3$. Steady-state levels of 1,25-$(OH)_2D_3$ in cells and the circulation are determined by a balance between 1,25-$(OH)_2D_3$ bioactivation and degradation (1). The rate-limiting step in the bioactivation pathway is catalyzed by 25-hydroxyvitamin D_3 1-hydroxylase (13, 14), and the kidney is the major site of synthesis of biologically active 1,25-$(OH)_2D_3$. Metabolic inactivation of 1,25-$(OH)_2D_3$ and conversion to water-soluble products are catalyzed by the C24 oxidation pathway (15). The initial step in this pathway involves the mitochondrial cytochrome P450C24 enzyme (*i.e.* 25-hydroxyvitamin D_3 24-hydroxylase, CYP24) (16). In the normal situation, CYP24 is expressed mainly in the kidney but can be substantially induced by 1,25-$(OH)_2D_3$ in this and many other tissues. The up-regulation of CYP24 in response to 1,25-$(OH)_2D_3$ constitutes an important negative feedback mechanism, whereby the hormone acts to regulate its ambient and cellular concentration. Hence, expression of CYP24 in kidney and other tissues serves a protective function in guarding against excessive levels of 1,25-$(OH)_2D_3$ that can be deleterious due to the attendant hypercalcemia. In addition, specific biological actions in bone have been reported for 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) (17), a metabolite whose cellular concentration is directed by regulation of *CYP24* gene expression. It is clear, therefore, that regulation of *CYP24* gene expression is important in the metabolism and function of metabolites from the vitamin D pathway.

Studies into the molecular mechanism by which 1,25-$(OH)_2D_3$ induces the *CYP24* gene are in progress (18–21). In the rat promoter, there are two functional VDREs located at −136/−150 and −244/−258 on the antisense strand (18). To date, this dual arrangement of VDREs is unique among the 1,25-$(OH)_2D_3$-responsive genes. The two VDREs conform to the classic sequence, each consisting of two direct repeats separated by a 3-bp spacer. Gel mobility shift analysis using antibodies has established that both VDREs bind heterodimeric complexes of VDR·RXR (18). Transcriptional synergy between the two VDREs has been demonstrated and is particularly

* This work was supported by research funds from the Australian Research Council and University of New Mexico Medical Trust Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131-5221. Tel.: 505-272-5791; Fax: 505-272-6587; E-mail: jomdahl@salud.unm.edu.

[1] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; EBS, Ets protein-binding site; CYP24, 25-hydroxyvitamin D_3 24-hydroxylase; VDR, vitamin D receptor; VDRE, vitamin D responsive element; GST, glutathione *S*-transferase; RXR, retinoid X receptor; FCS, fetal calf serum; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; bp, base pair.

evident at high levels of 1,25-$(OH)_2D_3$ (18). Indeed, the *CYP24* promoter appears to be the most responsive promoter to 1,25-$(OH)_2D_3$ so far identified with levels of induction of 20–70-fold being observed in transient assays (21). In our previous studies on the rat *CYP24* promoter, we unexpectedly observed that the proximal VDRE had greater transactivation ability than the distal VDRE, yet its affinity for the VDR·RXR complex in gel mobility shift assays was markedly lower (18). This finding raised the possibility of transcriptional cooperation between the VDR·RXR complex and nearby transcription factors (22). To investigate this possibility, we used computer analysis to identify both an Ets-binding site and a possible AP-1 site juxtaposed and downstream to the proximal VDRE. A possible gene regulatory role for an Ets-binding site has not been documented for the *CYP24* gene, whereas a functional Jun/Fos AP-1 site would be consistent with the observed action of TPA (12-*O*-tetradecanoylphorbol-13-acetate) to up-regulate CYP24 enzyme activity (23). Consequently, these two sites were evaluated for possible roles in facilitating the proximal VDRE functionality in mediating the action of 1,25-$(OH)_2D_3$ to induce expression of the *CYP24* gene.

EXPERIMENTAL PROCEDURES

Materials—1,25-$(OH)_2D_3$ was supplied by Hoffmann-La Roche. A Sequenase version 2.0 sequencing kit was purchased from U. S. Biochemical Corp. Oligonucleotides were synthesized by Bresatec (Adelaide, South Australia). The luciferase assay kit was from Promega (Madison, WI). Nickel-nitrilotriacetic acid chromatographic support and glutathione-agarose were from Qiagen GmbH (Germany).

Promoter-Luciferase Constructs and Expression Plasmids—Two oligonucleotide polymerase chain reaction primers engineered with *Kpn*I sites were employed to amplify −186 bp of the *CYP24* promoter sequence (encompassing the proximal VDRE at −150/−136) together with 74 bp of 5′-untranslated region using the pCYP24WT(−298)-Luc plasmid (18) as a template. The polymerase chain reaction product was cloned at the *Kpn*I site in pGL3-Basic vector containing the firefly luciferase reporter gene as described previously (24). Mutations in promoter-luciferase constructs (see Fig. 1*B*) in VDRE half-sites, EBS, and AP-1-like sites were introduced using the Quik-Change mutagenesis protocol (Stratagene, La Jolla, CA). The mutated constructs were sequenced to verify the desired mutations. pRSV-hVDR was generated by cloning human VDR cDNA sequence downstream of the Rous sarcoma virus promoter (21). The expression clones for human Ets-1, p42Ets-1, and Ets-2 are based on the pEFBOS vector and have been described previously (25). Human Fli-1 and PU-1 expression clones were kindly provided by Dr. Robert Hromas, Department of Medicine, Indiana University. An activated Ras (Ha-c-Ras) expression clone (pHO6T1), and expression clones for chicken p54-Ets-1 wild-type (c-Ets-1 Thr-38) and mutated cEts-1 (c-Ets-1 T38A) were kindly provided by Dr. Michael Ostrowski (Ohio State University) (26).

Maintenance and Transfection of Cells—Monkey kidney fibroblast COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). In preparation for electroporation, cells were grown in a 175-cm^2 flask to 60–70% confluency, washed once with phosphate-buffered saline, and removed by trypsinization. Transfections were performed by electroporation of cells (2×10^6) in 500 μl of ice-cold electroporation buffer as described previously (24), containing 250 μg of sheared salmon sperm DNA and 2 pmol of promoter luciferase construct. The electroporation of COS-1 cells (280 V and 960 microfarads) was performed using a Bio-Rad gene pulser. A reference plasmid, pRSV-βGal, was also included in the transfections to correct for variations in transfection efficiency. Following electroporation, the samples were placed on ice for 10 min and divided into two wells of a 6-well plate containing Dulbecco's modified Eagle's medium and 10% FCS. Cells were allowed to recover overnight, and medium was replaced with RPMI and 10% charcoal-stripped FCS. Cells were further treated with 1,25-$(OH)_2D_3$ (10^{-7} M) or ethanol carrier and incubated for 24 h prior to harvesting. Luciferase activity in cell lysates was determined as described previously (18). All cells used in transactivation studies were cotransfected with the VDR-expression vector pRSV-hVDR to compensate for a low endogenous VDR level in COS-1 cells (19).

Gel Mobility Shift Assays—Double-stranded oligonucleotides were synthesized to contain the EBS and AP-1-like sites (CYP24-EBS), a mutated core sequence of EBS (CYP24-mEBS), a mutated AP-1-like sequence (CYP24-mAP1), the proximal VDRE alone (CYP24-VDRE), or together with EBS as a composite oligonucleotide (VDRE + EBS). An oligonucleotide that encompassed a known Ets-1-binding site from T-cell receptor gene enhancer (TCRαEts-1) was employed as a control (27). Each double-stranded oligonucleotide was designed with *Sal*I and *Xho*I restriction enzyme sites at the 5′ or 3′ ends as shown.

```
5′-TCGACGCTGACTCCATCCTCTTC-3′
3′-GCGACTGAGGTAGGAGAAGAGCT-5′
```

OLIGONUCLEOTIDE CYP24-EBS

```
5′-TCGACGCTGACTCCAAAATCTTC-3′
3′-GCGACTGAGGTTTTAGAAGAGCT-5′
```

OLIGONUCLEOTIDE CYP24-mEBS

```
5′-TCGACGCGTTCTCCATCCTCTTC-3′
3′-GCGCAAGAGGTAGGAGAAGAGCT-5′
```

OLIGONUCLEOTIDE CYP24-mAP1

```
5′-TCGACGGCGCCCTCACTCACCTCGC-3′
3′-GCCGCGGGAGTGAGTGGAGCGAGCT-5′
```

OLIGONUCLEOTIDE CYP24-VDRE

```
5′-TCGACAGCCACATCCTCTGGAAC-3′
3′-GTCGGTGTAGGAGACCTTGAGCT-5′
```

OLIGONUCLEOTIDE TCRαEts-1

```
5′-TCGACGGCGCCCTCACTCACCTCGCTGACTCCATCCTCTTC-3′
3′-GCCGCGGGAGTGAGTGGAGCGACTGAGGTAGGAGAAGAGCT-5′
```

OLIGONUCLEOTIDE VDRE + EBS

Nuclear extracts were prepared from COS-1 cells (28). Each double-stranded oligonucleotide was labeled by end-filling with [α-^{32}P]dCTP using Klenow enzyme and purified by 12% polyacrylamide gel electrophoresis. Binding reactions for each assay contained 5 μg of nuclear protein, 1 μl of poly(dI-dC) in a final volume of 12 μl in Dignum buffer C (20 mM HEPES buffer, pH 7.6, 420 mM NaCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM EDTA, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) and were incubated on ice for 15 min. Radiolabeled probe (200,000 cpm) was added, and samples were incubated on ice for another 30 min. For gel-shift inhibition assays, a polyclonal antibody to Ets protein DNA binding domain (PAN-Ets) was employed, and a VDR monoclonal antibody designated IgG2b (Affinity BioReagents Inc., Neshanic Station, NJ) was used as a control. For supershift assays, a polyclonal antibody to Ets-1 (29) and a polyclonal antibody to Erg-1/Erg-2 (Santa Cruz Biotechnology Inc.) were employed. These antibodies were included in the binding reaction and incubated on ice for 15–30 min prior to addition of probe. Gel-shift competition assays were performed with unlabeled competitor oligonucleotide at molar excess concentrations (50–100-fold) by inclusion in the binding reactions. Retarded DNA nuclear protein complexes were resolved on a 4% nondenaturing polyacrylamide gel using precooled low ionic strength gel running buffer (0.5× TBE) at 4 °C. The gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at −80 °C.

Gel Mobility Shift Assays Using Purified Ets-1, VDR, and RXR—Recombinant full-length human Ets-1 (p54Ets-1) was produced in *Escherichia coli* as a carboxyl histidine-tagged fusion protein and purified on a nickel-nitrilotriacetic acid column. The tagged Ets-1 protein migrated as a single band with an apparent molecular mass of about 55 kDa in SDS-polyacrylamide gels. Recombinant VDR and RXR were produced in *E. coli* as N-terminal glutathione *S*-transferase (GST) fusion proteins. A clone pGEX-2T RXR, from Dr. George Muscat, University of Queensland, Australia, expressed GST-RXR, and a clone (pGEX-2T VDR) was created in our laboratory with human VDR cDNA cloned into pGEX2T in frame with GST sequence. The GST-VDR and GST-RXR fusion proteins were purified by affinity binding to glutathione-agarose beads.

DNA binding reactions contained recombinant Ets-1, GST-VDR, or GST-RXR with 50 ng of poly(dI-dC) in a final volume of 10 μl in binding buffer (20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 5 mM dithiothreitol, 1.0 mM EDTA, pH 8.0, 10% glycerol, and 100 μg/ml bovine serum albumin). Reactions were incubated at room temperature for 10 min. Radiolabeled probes (10,000 cpm) were added and samples further

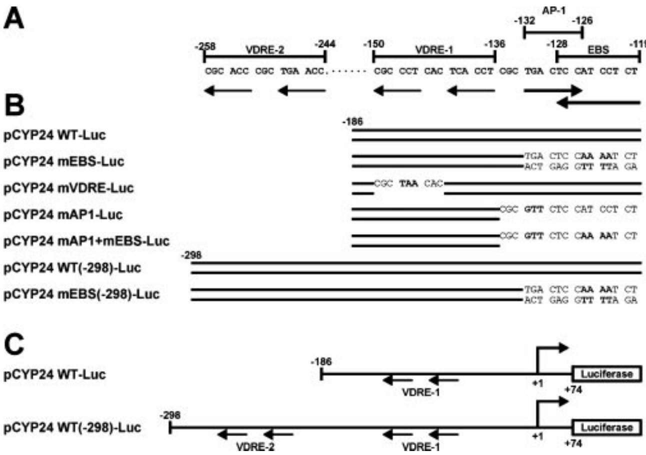

FIG. 1. **Mutations introduced into the EBS and AP-1-like sequence and VDRE located in the rat *CYP24* gene promoter.** *A*, sequence of native promoter encompassing the two VDREs, EBS and AP-1-like sequence. The *numbers above* the *lines* indicate the distance relative to the transcription initiation site. *Arrows below* the VDRE sequence indicate VDRE hexameric half-sites and their orientation, and *arrows below* EBS and the AP-1-like sequence indicate their orientation. *B*, various mutations (shown in *bold letters*) that were introduced in two different promoter length constructs (−186 and −298). *C*, diagrammatic representation of the wild-type *CYP24* promoter-luciferase construct of each promoter length (*i.e.* +74 to −186 and +74 to −298).

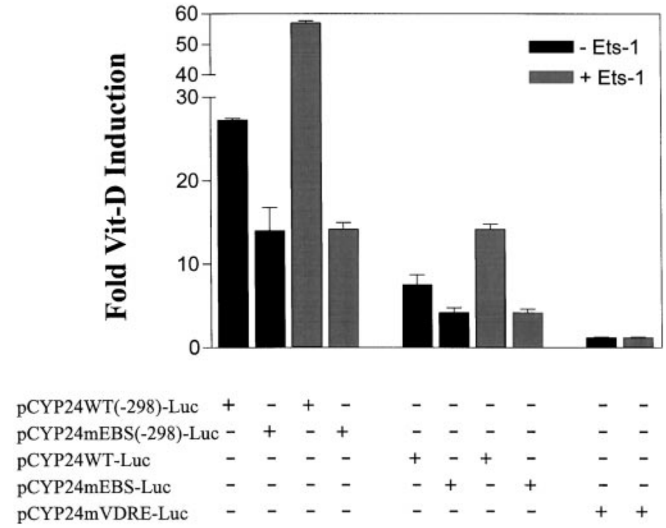

FIG. 2. **The role of EBS and the influence of Ets-1 protein on the 1,25-$(OH)_3D_2$-mediated transactivation of CYP24 promoter constructs.** COS-1 cells were cotransfected with pRSV-hVDR and either pCYP24WT(−298)-Luc, pCYP24mEBS(−298)-Luc, pCYP24mEBS-Luc or pCYP24mVDRE-Luc. As an internal control, cells were cotransfected with 5 μg of β-galactosidase expression vector, pRSV-βgal (see "Experimental Procedures"). Each construct was tested for 1,25-$(OH)_2D_3$-mediated (10^{-7} M) transactivation in the presence or absence of a transfected Ets-1 construct (10 μg). The levels of induction (*Fold Vit-D Induction*) are shown as the ratio of luciferase activity from 1,25-$(OH)_2D_3$-treated cells to that from untreated cells. Data presented are the average of three independent experiments ± S.D.

incubated at room temperature for 20 min. For supershift assays and competition binding assays, antibodies for Ets-1 and unlabeled competitor oligonucleotides, respectively, were included prior to addition of probe. Retarded complexes were resolved by 5% nondenaturing polyacrylamide gel electrophoresis. Association constants (K_a) for the binding of recombinant Ets-1 to CYP24-EBS and CYP24-mAP1 were determined by gel-shift assays. Increasing amounts of purified Ets-1 (0.125, 0.250, 0.500, and 1–4 μg) were incubated in a binding reaction with 1 ng of radiolabeled probe. The DNA protein complexes were resolved by 5% nondenaturing gel electrophoresis, dried, and exposed in the PhosphorImager (Molecular Dynamics). Ets-1-bound DNA complexes and unbound-probe were quantified using ImageQuant Software. The association constants, defined as the Ets-1 concentration at which 50% of the DNA probe was retarded, were determined by direct curve fitting (Sigma Plot, Jandel Scientific).

RESULTS

Identification of a Functional Ets Protein-binding Site (EBS) in the CYP24 Promoter—Previous expression studies (18) of the rat *CYP24* promoter suggested that 1,25-$(OH)_2D_3$-mediated transactivation at the proximal VDRE (−136/−150) required the participation of a nearby transcription factor(s). An examination of sequences downstream from this VDRE revealed a likely binding site for Ets transcription factors (5′-AGAGGATGGA-3′) at −119/−128 on the antisense strand (Fig. 1*A*). Members of the Ets transcription factor family bind to a common core motif (5′-GGA(A/T)-3′) with flanking sequence determining the specificity of binding. The site identified here with a 5′-GGAT-3′ core, strongly resembles known binding sites for Ets-1 (30). Also noted was a possible AP-1-like sequence (5′-TGACTCC-3′) at −132/−126, which differs from the consensus AP-1 site (5′-TGACTCA-3′) by one nucleotide and overlaps the Ets-binding site (EBS).

To evaluate whether the EBS contributes to 1,25-$(OH)_2D_3$-dependent *CYP24* promoter expression, the core EBS motif was mutated (5′-GGAT-3′ to 5′-TTTT-3′) in a luciferase-reporter construct that contained −298 bp of promoter sequence and both VDREs of the *CYP24* promoter. The wild-type and mutated-EBS promoter constructs are designated pCYP24WT(−298)-Luc and pCYP24mEBS(−298)-Luc, respectively (Fig. 1, *B* and *C*). In response to 1,25-$(OH)_2D_3$, the wild-type construct gave a 27.2-fold level of induction, but this was reduced to 14.2-fold when the EBS was inactivated (Fig. 2). The importance of the EBS in sustaining the 1,25-$(OH)_2D_3$-mediated transactivation of the *CYP24* promoter constructs was explored further by cotransfection studies with an Ets-1 expression vector. Cells containing the pCYP24WT(−298)-Luc displayed more than a doubling of activity (58.2-fold) when cotransfected with Ets-1 (Fig. 2). The mutant EBS construct in the presence of Ets-1 gave the same activity (14.5-fold) as observed for this mutated construct in the absence of Ets-1 (Fig. 2). It was evident from these studies, therefore, that the EBS was critical for maximal up-regulation of the *CYP24* promoter. Due to the location of the EBS in the region downstream of the proximal VDRE, subsequent studies were conducted using constructs that contained only the proximal promoter element and adjacent EBS and AP-1-like sequences.

The single VDRE construct (−186 bp of promoter sequence; pCYP24WT-Luc) expressed a 7.6-fold level of induction (Fig. 2), which is about 28% of the value obtained with the construct containing both functional VDREs (*i.e.* pCYP24WT(−298)-Luc). This activity for the proximal VDRE is about 2-fold higher than the contribution made by the more distal VDRE (data not shown), which reflects the cooperative synergism between the two VDREs in the rat *CYP24* promoter (18). Mutation of the EBS in the proximal VDRE construct (*i.e.* pCYP24mEBS-Luc) resulted in a substantial loss in transactivation activity (7.6- to 4.2-fold) that was qualitatively similar to results obtained using the native two-VDRE construct (*i.e.* pCYPWT(−298)-Luc) (Fig. 2). Transfected Ets-1 plasmid was ineffective in stimulating transactivation of the mutated EBS construct (*i.e.* pCYP24mEBS-Luc, 4.3-fold induction), whereas induction was increased from 7.6- to 14.0-fold in cells containing the wild-type construct with an intact EBS (*i.e.* pCYP24WT-Luc) (Fig. 2). In the absence of added 1,25-$(OH)_2D_3$, the basal level of expression of the wild-type construct was not affected by either EBS inactivation or overexpression of Ets-1 (data not shown).

Mutagenesis of the VDRE alone (pCYP24mVDRE-Luc, Fig.

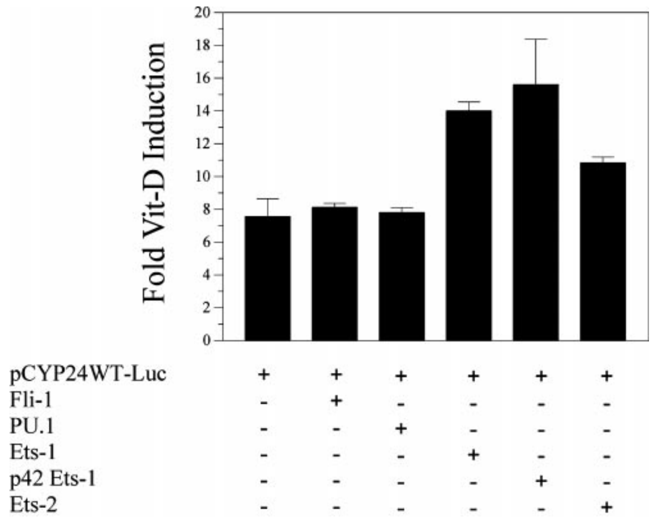

FIG. 3. **Activity of specific Ets proteins in the 1,25-$(OH)_2D_3$-mediated transactivation of the CYP24 promoter.** COS-1 cells were cotransfected (see Fig. 2 and "Experimental Procedures") with pCYP24WT-Luc and one of five different Ets constructs (10 μg each) and tested for 1,25-$(OH)_2D_3$-mediated transactivation of the CYP24 promoter construct. Data are the average of three independent experiments ± S.D.

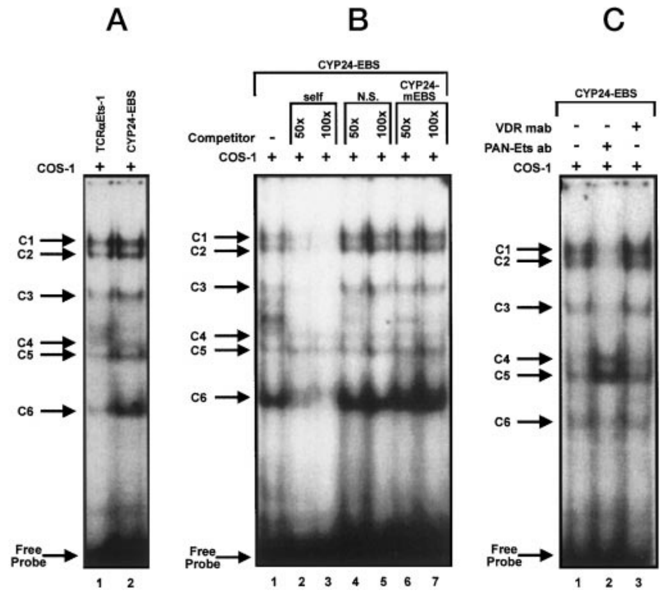

FIG. 4. **Binding of nuclear proteins to the EBS.** *A*, double-stranded oligonucleotides to EBS (CYP24-EBS) and a control EBS (TCRαEts-1) were labeled by end-filling with [α-^{32}P]dCTP and incubated with nuclear extracts from COS-1 cells. The major retarded complexes are *arrowed* and *numbered* as *C1 to C6*. Free probe is *arrowed* at the *bottom* of the gel. *B*, the formation of complexes with labeled CYP24-EBS in *A* was competed with three unlabeled probes (*i.e.* self, nonspecific probe (CYP24-VDRE) or CYP24-mEBS). *C*, for neutralization assays, nuclear extracts were incubated with (+) or without (−) polyclonal antibodies for Ets (PAN-Ets, *lane 2*) or VDR monoclonal antibody (VDRmab, *lane 3*) prior to addition of the labeled probe CYP24-EBS.

1*B*) lowered induction of the wild-type construct to 1.2-fold (Fig. 2) confirming that this is the only functional VDRE present in the −186 bp of promoter sequence as reported previously (24). Overexpression of Ets-1 was also investigated on the construct pCYP24mVDRE-Luc. In this situation, there was no effect on expression in the presence of hormone (Fig. 2) confirming that Ets-1 cannot transactivate in the absence of a functional VDRE. The data in Fig. 2 provide evidence for transcriptional synergy between the EBS and VDRE. For example, the 1,25-$(OH)_2D_3$ induction of the wild-type promoter construct pCYP24WT-Luc in the presence of Ets-1 was 14.0-fold, which is greater than the sum of the individual contributions from VDRE (4.2-fold) and EBS (1.2-fold). When the constructs pCYP24WT-Luc and pCYP24mEBS-Luc were tested at a lower 1,25-$(OH)_2D_3$ concentration (*i.e.* 10^{-10} M), a decrease in induction occurred for the wild-type construct (from 7.6 ± 1.1- to 2.8 ± 0.3-fold), and no induction was observed for the mEBS construct. For comparison, the induction of the −298 construct (pCYP24WT(−298)-Luc) was also examined. At this low concentration of 1,25$(OH)_2D_3$, induction was lowered from 27.2 ± 1.8- to 3.7 ± 0.5-fold, and mutagenesis of the EBS led to the loss of induction (1.5 ± 0.25-fold). It is clear, therefore, that the EBS is critical for the function of the proximal VDRE, especially when hormone is near physiological concentration.

Specificity of Transactivation by Ets Proteins—The role of EBS in stimulating *CYP24* promoter activity was evaluated in the transactivation assay using various Ets proteins. Ets family members can be classified according to their homology to Ets-1 (31). Class I members such as Ets-1 and Ets-2 have extensive overall sequence similarity (30). Erg-1 and Fli-1, class II members, show homology to Ets-1 both in their N-terminal transactivation and C-terminal DNA binding domains, whereas class III members such as PU.1 show homology only in the DNA binding domain (30). We investigated the ability of a representative member from each of the three classes (human Ets-1, Fli-1, and PU.1) to transactivate the wild-type *CYP24* promoter in COS-1 cells. Neither Fli-1 nor PU.1 further increased the 7.6-fold level of 1,25-$(OH)_2D_3$ induction observed with the pCYP24WT-Luc construct (Fig. 3). By contrast, Ets-1 elevated 1,25-$(OH)_2D_3$ induction of the wild-type construct to 14.0-fold (Fig. 3). The inability of Fli-1 or PU.1 to bind the EBS was not investigated.

The transactivation activity of p42Ets-1, an alternatively spliced form of Ets-1 (32), and Ets-2, both class I members of the Ets family, were also evaluated in overexpression studies. The activity of p42Ets-1 was comparable to Ets-1, where it stimulated the wild-type level of induction from 7.6- to 15.6-fold, whereas Ets-2 was less active (7.6- to 10.8-fold) (Fig. 3). It was further shown that the observed transactivation activities of p42Ets-1 and Ets-2 were completely abrogated when the EBS site in the promoter was mutated. Similar to Ets-1, neither p42Ets-1 nor Ets-2 altered basal expression of the wild-type construct or expression of the mutant VDRE construct (pCYP24mVDRE-Luc) in the presence or absence of hormone (data not shown).

EBS Binds Multiple Proteins from COS-1 Cell Nuclear Extracts—To investigate nuclear protein binding to the EBS, gel mobility shift analysis was carried out using COS-1 cell nuclear extracts and the oligonucleotide probe CYP24-EBS. Six nuclear-protein complexes (C1–C6) were consistently detected together with an inconsistently appearing band(s) between C3 and C4 (Fig. 4*A*). A similar profile of protein binding was observed with a control Ets-1 probe (TCRαEts-1) from the T-cell receptor α-gene enhancer (27), but the binding was weaker than for CYP24-EBS (Fig. 4*A*). Mutagenesis of the core region in CYP24-mEBS abolished the binding of all complexes except C4 and C5 (data not shown). Self-competition experiments inhibited formation of complexes C1–C3 and C6 (Fig. 4*B*). In contrast, competition experiments with the nonspecific probe CYP24-VDRE and mutated probe CYP24-mEBS had no effect on complexes C1–C3 and C6. However, the nonspecific and mutated probes were partially active in preventing formation of the apparent nonspecific complexes migrating between C3 and C6 (Fig. 4*B*). To characterize further the Ets proteins

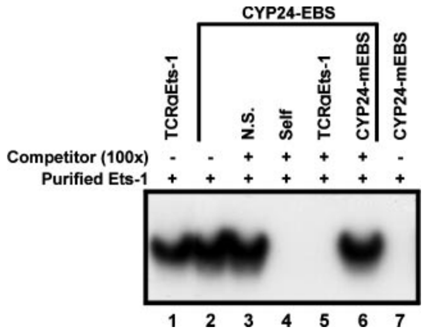

FIG. 5. **Recombinant human p54Ets-1 binds to CYP24-EBS oligonucleotide.** Double-stranded oligonucleotides to EBS (CYP24-EBS), mutated EBS (*CYP24-mEBS*), and a control EBS (*TCRαEts-1*) were labeled by end-filling with [α-^{32}P]dCTP and incubated with purified Ets-1. A single complex was retarded on TCRαEts-1 (*lane 1*) and CYP24-EBS (*lane 2*), but no complex was observed with CYP24-mEBS (*lane 7*). The retarded complex on CYP24-EBS was competed with 100-fold molar excess of nonspecific (*N.S.*) competitor (*lane 3*), self-competitor (*lane 4*), TCRαEts-1 (*lane 5*), and CYP24-mEBS (*lane 6*).

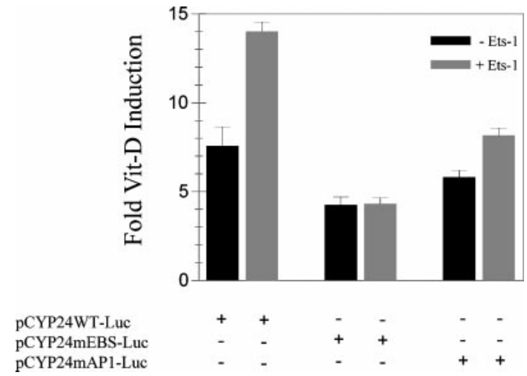

FIG. 6. **CYP24-AP1-like sequence is the flanking sequence for EBS.** COS-1 cells were cotransfected with either pCYP24WT-Luc, pCYP24mEBS-Luc, or pCYP24mAP1 (see Fig. 2 and "Experimental Procedures"). CYP24 promoter constructs were tested for 1,25-$(OH)_2D_3$-mediated transactivation in the presence or absence of transfected Ets-1 expression clone (10 μg). Data presented are the average of three independent experiments ± S.D.

present in complexes C1–C6, gel mobility shift experiments employing various antibodies were conducted. A neutralizing polyclonal antibody (PAN-Ets) that interferes with the binding of Ets proteins to the core sequence markedly reduced the formation of complexes C1–C3 but only weakly affected C6 and had no effect on C4 or C5 complex formation (Fig. 4*C*). Control VDR monoclonal-neutralizing antibody was without effect (Fig. 4*C*). An Ets-1-supershifting polyclonal antibody (29) functioned to supershift complexes C1, C2, and C3 (data not shown). However, migration of complexes C1–C6 was not altered by either a supershifting polyclonal antibody to the Ets proteins Erg-1/Erg-2 or an Ets-2 polyclonal supershifting antibody (data not shown). It can be concluded from the antibody gel mobility shift data that EBS binds complexes C1–C3 and C6, which are related immunologically to Ets-1. However, complexes C4 and C5 appeared to be nonspecifically bound to the CYP24-EBS probe. We have shown directly that purified Ets-1 binds to CYP24-EBS but not to CYP24-mEBS (Fig. 5). The complex was competed by self and TCRαEts-1 but not with a nonspecific oligonucleotide or CYP24-mEBS. The recombinant Ets-1 complex was found to comigrate with complex C3 (data not shown, see Fig. 4 for binding complexes migration patterns).

An AP-1-like Sequence Overlaps the EBS—A possible AP-1-like site (−132/−126) with a 3-base overlap at the 3′-end of the EBS on the antisense strand (Fig. 1*A*) was evaluated extensively for functionality. Protein binding to this site was not observed in gel mobility shift assays with either purified c-Fos or c-Jun protein or with nuclear extracts from phorbol ester-treated cells. Furthermore, overexpression of c-Fos and c-Jun failed to alter CYP24 promoter expression (data not shown). Therefore, rather than functioning as a classical AP-1 site, the possibility was considered that the AP-1-like sequence represents extended EBS-flanking sequence. To investigate this hypothesis, both the putative AP-1-like sequence and the EBS were mutated (*i.e.* pCYP24mAP1-Luc and pCYP24mEBS-Luc) and compared for transactivation activity. Induction with 1,25-$(OH)_2D_3$ (7.6-fold) was lowered to 5.8-fold when the AP-1-like site was mutated, whereas mutagenesis of the core EBS reduced induction to 4.2-fold (Fig. 6). A double mutation of both sites gave a similar activity level to that seen with the core EBS mutation (data not shown). Hence, mutagenesis of the AP-1-like sequence does not further inhibit induction of the construct with the inactivated EBS core motif. On this basis, it was concluded that the 5′-TGA-3′ sequence constitutes flanking sequence that is important for maximal EBS activity. Presumably, the remaining sequence of the AP-1-like site also contributes to the EBS-flanking sequence (Fig. 1*A*).

To investigate further the AP-1-like sequence, transactivation experiments with Ets-1 were performed. When Ets-1 was overexpressed, induction by 1,25-$(OH)_2D_3$ of the wild-type construct was 14.0-fold, which was reduced to 8.2-fold with pCYP24mAP1-Luc (Fig. 6). By comparison, mutagenesis of the EBS core gave a 4.2-fold level of induction, and overexpressed Ets-1 was ineffective (Fig. 6). Hence, alteration of the flanking sequence substantially reduces transactivation by Ets-1. Consistent with the transactivation studies, gel mobility shift assays with CYP24-mAP1 showed reduced binding of complexes C1–C3 and C6 (data not shown). Association constants (K_a) were determined in the gel mobility shift assays for the binding of recombinant Ets-1 to CYP24-EBS and CYP24-mAP1 probes. By using the wild-type probe, the K_a value for Ets-1 was 1.2 ± 0.12 × 10^6 M^{-1}, which was decreased by ~25% to 0.89 ± 0.09 × 10^6 M^{-1} with probe containing the mutated flanking sequence. This decrease in K_a for the CYP24-mAP1 probe supports further the proposal that the 5′-TGA-3′ sequence from the AP-1-like site is important for optimal binding of endogenous Ets proteins. Based upon the collective data, it was evident that the AP-1-like site was not functional. Therefore, subsequent studies were directed at delineating the detailed function of the EBS to facilitate the transactivation activity of 1,25-$(OH)_2D_3$ on the rat *CYP24* promoter.

Ets-1 and VDR·RXR Form a Ternary Complex on DNA—Although the identity of the Ets proteins that bind to the EBS *in vivo* are not known, we have demonstrated that this site can respond to Ets-1 in transactivation experiments and bind recombinant Ets-1. On this basis, we investigated further the mechanism of Ets-1 action. By using gel mobility shift analysis, we showed that purified Ets-1, VDR, and RXR form a complex on an oligonucleotide (VDRE + EBS) that spans both the proximal VDRE and the EBS. When Ets-1 was incubated with the probe, a single complex C-I was retarded (Fig. 7, *lane 2*), and the amount of this complex was reduced by addition of VDR and RXR with the appearance of a new slower mobility complex C-II (*lane 3*). Complex C-II was also generated when VDR and RXR were added to DNA prior to Ets-1 (data not shown). The complex generated by VDR and RXR alone was intermediate in size between complexes CI and CII (Fig. 7, *lane 4*). Formation of the complexes C-I, C-II, and VDR-RXR was suppressed (*lane 5*) in the presence of excess unlabeled self-competitor oligonucleotide (VDRE + EBS), which established the specificity of these complex formations. To demonstrate that C-II results from the formation of a ternary complex between Ets-1, VDR, RXR, and the VDRE + EBS probe, compe-

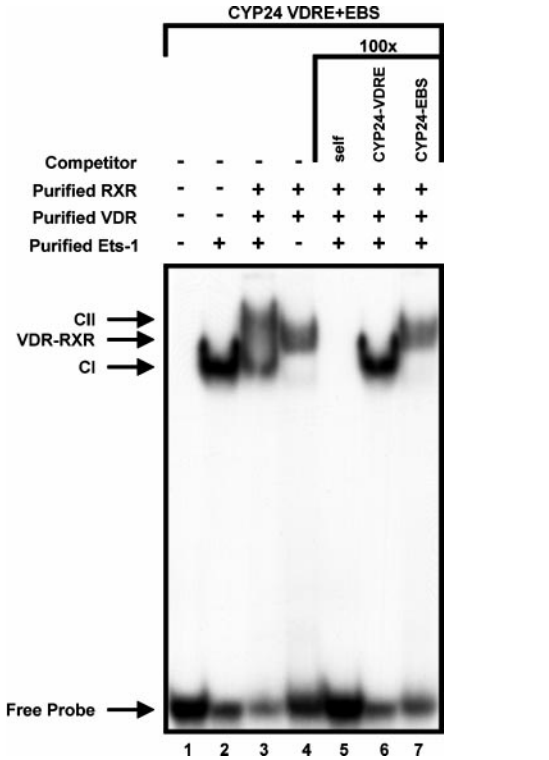

FIG. 7. **Purified VDR, RXR, and Ets-1 form a ternary complex with VDRE + EBS oligonucleotide.** A double-stranded oligonucleotide encompassing VDRE and the EBS site (VDRE + EBS) was labeled by end-filling with [α-^{32}P]dCTP and incubated either with purified Ets-1 (*lane 2*) or VDR and RXR (*lane 4*), and the specific complexes are indicated by *arrows.* In *lanes 3, 5, 6*, and 7, the probe was preincubated with purified Ets-1 for 15 min followed by addition of VDR and RXR. The specific complexes retarded (*arrowed*) were for Ets-1 alone (*CI*), VDR-RXR complex, and a unique complex (*CII*) resulting from the addition of VDR and RXR. CII was competed by 100-fold molar excess of self-competitor (*lane 5*), CYP24-VDRE alone (*lane 6*), and CYP24-EBS alone (*lane 7*). This experiment was repeated three times, and the same result was obtained.

tition experiments were performed. The competition by 100-fold molar excess of an oligonucleotide corresponding to the VDRE (CYP24-VDRE) resulted in conversion of complex C-II into complex C-I (*lane 6*), whereas competition with an excess of an oligonucleotide corresponding to the EBS site (CYP24-EBS) resulted in conversion of complex II to the VDR·RXR complex (*lane 7*). This confirmed that the slower migrating complex II contains Ets-1 and the VDR·RXR complex and that both binding sites (VDRE and EBS) are required for this ternary complex formation. In these experiments, we consistently observed a preferential binding of VDR·RXR to the preformed Ets-1·VDRE + EBS complex (C-I) rather than to free VDRE + EBS probe (compare *lane 3,* where VDR-RXR was added to preformed Ets-1·VDRE + EBS complex, and *lane 4,* where VDR-RXR was added to free VDRE + EBS). A similar observation of preferential binding was made in titration experiments using different concentrations of VDR·RXR and fixed Ets-1 as well as in the reverse experiments (data not shown). This finding raises the possibility of a physical interaction between VDR·RXR and Ets-1, the details of which require further investigation.

The EBS in the CYP24 Promoter Is a Ras/Raf-responsive Element—The Ras/Raf signaling pathway plays a key role in the activation of Ets-1 through phosphorylation of threonine residue 38 (26, 33). We investigated whether a similar Ras-mediated action is involved in the transactivation of the *CYP24* promoter by exogenous Ets-1. Initial tests used wild-type cEts-1 (the p54 form of chicken Ets-1) and a mutant form where

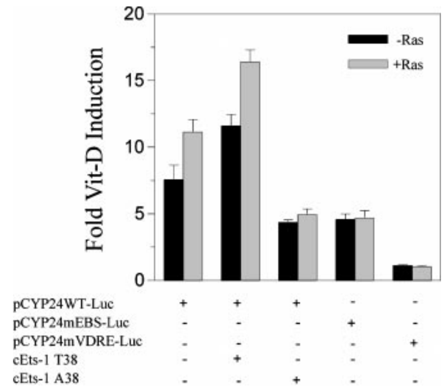

FIG. 8. **Ras and cEts-1 Thr-38 stimulate 1,25-$(OH)_2D_3$ directed induction of the CYP24 promoter.** COS-1 cells were cotransfected with either pCYP24WT-Luc, pCYP24mEBS-Luc, or pCYP24mVDRE-Luc together with 10 μg of wild-type c-Ets-1 Thr-38 or mutated c-Ets-1 T38A expression clones (see Fig. 2). CYP24 promoter constructs were tested for 1,25-$(OH)_2D_3$-mediated transactivation in the presence or absence of transfected Ras expression clone (10 μg) (see "Experimental Procedures"). Data presented are the average of three independent experiments ± S.D.

threonine 38 was mutated to an alanine (*i.e.* T38A) (26). Overexpression of cEts-1 increased 1,25-$(OH)_2D_3$ induction of pCYP24WT-Luc from 7.6- to 11.6-fold (Fig. 8). When the EBS was mutated, there was no effect of overexpressed cEts-1 on induction (data not shown). By contrast, overexpression of the T38A mutant reduced the level of induction from 7.6- to 4.4-fold, a level comparable to that observed when the EBS was mutated (see Fig. 8). These results were interpreted to mean that the mutant protein competes with endogenous Ets proteins for binding to EBS but is unable to enhance transactivation. Therefore, these experiments establish threonine 38 as a critical residue for cEts-1 transactivation and imply that the Ras pathway is involved in cEts-1 function.

When Ras was overexpressed, the level of induction of the pCYP24WT-Luc construct was increased from 7.6- to 11.2-fold, and this was further increased to 16.4-fold when wild-type cEts-1 was also transfected (Fig. 8). The Ras-dependent transactivation was completely inhibited when the EBS was mutated (Fig. 8). When the T38A mutant cEts-1 was overexpressed, the Ras-mediated level of induction was lowered to 4.9-fold, which corresponded to the level of induction observed with the mutated EBS (Fig. 8). When the VDRE was mutated (*i.e.* pCYP24mVDRE-Luc), there was no effect of exogenous Ras on either basal expression or 1,25-$(OH)_2D_3$-dependent induction (Fig. 8) in keeping with the conclusion that the VDRE is critical for EBS function.

Further evidence for the involvement of endogenous Ras activity was obtained with manumycin A, an inhibitor of Ras farnesylation (35). The inhibitor was not effective at 10 μM, but when tested at 50 μM, 1,25-$(OH)_2D_3$-dependent induction of pCYP24WT-Luc was lowered from 7.7- to 4.1-fold, and the same result was observed with inhibitor at 100 μM. This level of expression was equivalent to that observed with mutated EBS (*e.g.* see Fig. 2). Additionally, there was no effect of manumycin A on induction when the EBS was inactivated. Hence, in these cells, the Ras signaling pathway appears to be entirely responsible for Ets protein activation.

Ets-1 Increases Basal Expression in Presence of a VDR Homodimer—Ets-1 was unable to alter basal expression even in the presence of overexpressed Ras. In a related manner, we have recently shown that the binding of VDR·RXR inhibits basal expression of the *CYP24* promoter possibly through the action of a corepressor (24). The binding or activation of Ets-1 could be prevented by the putative corepressor. As seen in Fig.

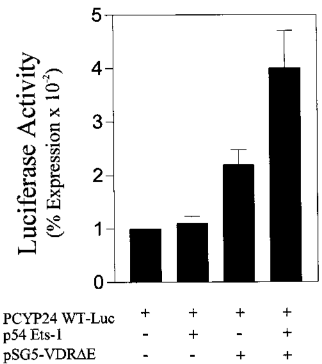

FIG. 9. **Ets-1 increases basal expression of *CYP24* promoter expression in the presence of VDRΔE.** COS-1 cells were cotransfected with either pCYP24WT-Luc and pSG5-VDRΔE (1 μg)(24) or p54-Ets-1 (10 μg) (see Fig. 2 and "Experimental Procedures"), but they were not treated with 1,25-$(OH)_2D_3$ due to the measurement of basal activity. Luciferase activity of pCYP24WT-Luc is taken as 100%, and activities are expressed relative to this value. Data presented are the average of three independent experiments ± S.D.

9, however, basal expression was increased when cells were transfected with a mutant form of VDR (VDRΔE) that strongly binds to the VDRE as a homodimer (24). Overexpression of Ets-1 (together with the mutant VDR) consistently resulted in a further increase in basal expression (Fig. 9). This result indicates that the presence of unliganded VDR·RXR on the promoter, but not VDRΔE, prevents the action of Ets-1. Based upon the current results and our previous work (24), we favor a model in which the putative corepressor-complex bound to unliganded VDR·RXR is responsible for EBS inactivity during basal conditions.

DISCUSSION

Previous results from our laboratory (18) and others (19, 20) have shown that the first 300 bp of rat *CYP24* promoter sequence is required for the 1,25-$(OH)_2D_3$ regulation of gene expression. The hormone-initiated transactivation is mediated through two VDREs located at −136/−150 and −244/−258. Interestingly, the proximal VDRE supports transactivation to a greater extent than does the distal VDRE. The molecular basis of this observation was investigated in the current study by evaluating sites immediately adjacent to the proximal VDRE that could function to alter VDR-RXR-initiated transactivation. We subsequently identified a possible AP-1 site (−132/−126) and a consensus Ets-binding site (−128/−119) that were juxtaposed to the proximal VDRE in both the rat and human (34) *CYP24* promoters. The molecular role of the two binding sites in promoting the gene-regulatory action of 1,25$(OH)_2D_3$ was the current project focus.

The presence of a functional AP-1 site in the promoter of the *CYP24* gene has been suggested from various studies in which 24-hydroxylase enzyme activity was shown to be stimulated in response to treatment with the phorbol ester TPA (23, 36, 37). The role of TPA as a potent stimulator of protein kinase C (PKC) activity is well documented, and many of its actions are expressed through the Jun/Fos AP-1 system (38, 39). Also applicable to the current study is the observation that composite AP-1/Ets sites can function in a cooperative fashion to regulate gene expression (40). Consequently, the possibility was considered that the AP-1 sequence near the proximal VDRE could be involved in the action of phorbol esters to stimulate expression of the 24-hydroxylase enzyme. However, the AP-1-like sequence site was found to not be a functional AP-1 site, but it was demonstrated instead to constitute a 3′-flanking sequence of the EBS on the antisense strand. Therefore, if an AP-1 site is involved in mediating the up-regulation of *CYP24* gene expression of TPA, it must be located in another region of the promoter (41).

The identified Ets-binding site (EBS) was located approximately two turns of the helix downstream from the proximal VDRE. It was found to contain a 5′-GGAT-3′ core sequence that has been shown by other investigators (30) to provide specificity for the binding of Ets-1 and other closely related Ets proteins. In the corresponding position in the human *CYP24* promoter, the same EBS is present although it differs by one nucleotide in the flanking region (33). The functionality of the EBS in the rat CYP24 promoter was evaluated by transient expression analysis of constructs that contained both VDREs or only the proximal VDRE. In both instances, mutagenesis of the core sequence within the EBS did not alter basal expression of the construct but lowered the level of 1,25-$(OH)_2D_3$ induction. Participation of an Ets protein in the induction mechanism was strongly supported by the finding that overexpression of either Ets-1, p42Ets-1, or Ets-2 markedly increased the transactivation response to 1,25-$(OH)_2D_3$ in which stimulation by the Ets proteins was totally dependent upon an intact EBS. For example, transactivation by Ets-1 resulted in a 14-fold induction of the proximal VDRE construct, which was reduced by 70% when the EBS was mutated. By comparison, promoter induction was not affected by overexpression of the class II Fli-1 or class III PU.1 proteins. It was concluded, therefore, that specific Ets proteins, closely related to Ets-1 and representing class I members (31), can stimulate 1,25-$(OH)_2D_3$-dependent induction in transactivation experiments. The inactivity of Fli-1 and PU.1 could reflect their inability to bind the EBS in the *CYP24* promoter. The stimulatory action of the alternatively spliced isozyme p42Ets-1 is interesting, since this protein failed in other studies to transactivate a functional EBS in the human GM-CSF promoter.[2] The distribution and relevance of this isozyme to *CYP24* gene regulation in kidney and other tissues remains unknown.

The EBS in the *CYP24* promoter bound six protein complexes in the COS-1 cell nuclear extracts as determined by gel mobility shift analysis. Formation of four of the complexes (C1–C3, C6) was inhibited in self-competition experiments. The three larger complexes (*i.e.* C1–C3) were supershifted with Ets-1-specific polyclonal antibody and neutralized with a PAN-Ets antibody. Based upon gel mobility shift studies with pure protein, complex C3 appeared to be an Ets-1 complex. However, the identity of this complex and the other two higher molecular weight complexes remains to be conclusively determined. The fourth and smallest Ets complex (*i.e.* C6) displayed lower specificity as it did not supershift, and neutralization of its binding to EBS with the PAN-Ets competitive antibody was weak. Recently, another Ets protein with similarities to Ets-1 was identified in kidney and other tissues (42). Such observations emphasize the challenge in identifying a specific endogenous Ets protein(s) that stimulates *CYP24* promoter induction. A similar problem has arisen in other studies where *in vitro* binding of multiple Ets proteins to a single functional EBS has been observed (25, 43).

Mutagenesis of the EBS and VDRE motifs in transactivation experiments provided evidence for transcriptional synergism between the two sites in response to hormone. This cooperation may reflect a direct interaction between the Ets-1 type protein and liganded VDR·RXR complex on the *CYP24* promoter. Although this remains to be demonstrated, numerous other studies have established synergy between Ets-1 and transcription factors on both cellular and viral promoters (44–52), between

[2] P. P. Dwivedi, J. L. Omdahl, I. Kola, D. A. Hume, and B. K. May, unpublished data.

Ets-1 and a partner protein (43–45, 47, 49), or an indirect interaction of Ets via the coactivator CBP (53). Ets-1 possesses internal negative regulatory domains that hinder its monomeric binding to DNA (54), and therefore, the interaction with a neighboring protein would facilitate binding to the promoter (55, 56). The lack of such an interaction would explain why the EBS is inactive when the proximal VDRE is mutated in the *CYP24* promoter.

Ets proteins have been identified as key nuclear mediators of Ras/Raf action. There is recent evidence that the ability of Ets-1 to transactivate promoters is dependent upon its phosphorylation at a conserved threonine (residue 38) by the Ras/Raf pathway (26). However, phosphorylation of Ets-1 is apparently not required on some Ets-1-responsive promoters (26). Endogenous Ras was demonstrated in the current study to be important for Ets protein activation. Ras overexpression substantially enhanced the 1,25-$(OH)_2D_3$ induction of the *CYP24* promoter, and the Ras response was totally dependent on an intact EBS. Furthermore, Ras overexpression potentiated transactivation by Ets-1, but Ras was ineffective with the T38A mutant Ets-1 protein in which threonine 38 was not available for phosphorylation. Interestingly, expression of the T38A mutant protein reduced the level of 1,25-$(OH)_2D_3$-induced expression to the activity observed by a *CYP24* promoter construct in which the EBS was mutated. It is apparent from studies with the *CYP24* proximal promoter that mutant T38A Ets protein can bind to the EBS and not be transcriptionally active. Therefore, Ets-enhanced transactivation at the proximal *CYP24* promoter region appears to occur through its phosphorylated threonine, which may be necessary for the interaction of the coregulator with VDR·RXR or the transcription machinery. In a recent report (57), Ras transformation of human keratinocytes resulted in the inactivation of VDR·RXR complex formation, but the relationship of this finding to the Ras-mediated activation of 1,25$(OH)_2D_3$ induction in the present work remains to be established.

An intriguing observation in the current study involved the lack of response for the EBS in the absence of 1,25-$(OH)_2D_3$ ligand. There is evidence for the binding of a repressor complex to unliganded VDR·RXR (24), and this could prevent either Ets protein binding or its facilitation of a transactivation response. The current experiments in which a VDR mutant (VDRΔE) allowed Ets-1 to increase basal expression demonstrated further the repressive nature of the unliganded VDR·RXR complex and the requirement of a partner protein for Ets-1 functionality.

The contribution of the EBS site to 1,25-$(OH)_2D_3$ induction was particularly noticeable when the concentration of 1,25-$(OH)_2D_3$ was lowered from the non-limiting concentration used routinely in this work (10^{-7} M) to a more physiological concentration of 10^{-10} M. At this concentration of 1,25-$(OH)_2D_3$, activity of the proximal VDRE was almost entirely dependent on a functional EBS. This result demonstrates the important role played by the EBS at or near physiological concentrations of 1,25-$(OH)_2D_3$. Under such conditions, the activity of limiting amounts of bound liganded VDR·RXR to the proximal VDRE may be critically dependent on a cooperative interaction with the nearby Ets protein. Regulation of *CYP24* gene expression by 1,25-$(OH)_2D_3$ constitutes a feedback control mechanism whereby 1,25-$(OH)_2D_3$ regulates its own ambient concentration. Induced levels of CYP24 would result in the increased oxidation of 1,25-$(OH)_2D_3$ to water-soluble products and subsequent excretion (16). A synergistic response between the VDREs at higher levels of hormone would ensure maximal promoter activity and the rapid removal of excess 1,25-$(OH)_2D_3$. At lower hormone levels, in contrast, promoter expression would expectantly be weaker and result in lowered CYP24-mediated degradation of 1,25$(OH)_2D_3$ due to the dominant action of the proximal VDRE in which the EBS-binding proteins play a key role.

The present work represents the first report where Ets proteins have been shown to contribute to 1,25-$(OH)_2D_3$-dependent promoter activity. Since several Ets proteins are coexpressed in most tissues (42, 58), it remains a challenge to determine which of these is required for activity on the *CYP24* promoter. We demonstrated in COS-1 cells that signaling by Ras is essential for Ets-1 activation. However, PKC activity may also be important for *CYP24* promoter expression (23, 36, 37). There are reports that 1,25-$(OH)_2D_3$ stimulates PKC activity (59, 60), and recently, a plasma membrane VDR was identified that mediated PKC activation in response to 1,25-$(OH)_2D_3$ (61, 62). This finding gives rise to the interesting possibility that *CYP24* promoter expression in response to 1,25-$(OH)_2D_3$ may be controlled at one or both extranuclear sites, involving PKC and Ras/Raf signaling. In that regard, it is evident from this study that the combined action of 1,25-$(OH)_2D_3$ and mitogen-activated protein kinase pathway activity can facilitate a rapid response of the *CYP24* gene to 1) tightly regulate ambient 1,25-$(OH)_2D_3$ levels and, thereby, prevent elevations in the hormone and calcium that would be toxic to critical organ functions, and 2) modulate synthesis of 24-hydroxylated metabolites that express important biological functions. Whether 1,25-$(OH)_2D_3$ can directly activate Ras in the *CYP24* promoter system remains an interesting issue for future studies.

Acknowledgments—We are particularly grateful to Professor Jacques Ghysdael for the Ets-1-specific polyclonal antibody, Dr. Keith Shearwin for advice with K_a determinations, Dr. Ross Thomas for helpful discussions, and Chris Matthews for excellent technical assistance.

REFERENCES

1. Jones, G., Strugnell, S. A., and DeLuca, H. F. (1998) *Physiol. Rev.* **78,** 1193–1231
2. Christakos, S., Pandya, M. R., Wernyj, R. P., and Yang, W. (1996) *Biochem. J.* **316,** 361–371
3. Hewison, M., and O'Riordan, J. L. H. (1997) in *Vitamin D* (Feldman, D., Glorieux, F. H., and Pike, J. W., eds) pp. 447–462, Academic Press, San Diego
4. Issa, L. L., Leong, G. M., and Eisman J. A. (1998) *Inflamm. Res.* **47** 451–475
5. Norman, A. W. (1998) *J. Bone Miner. Res.* **13,** 1360–1369
6. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
7. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M., (1996) *Cell* **83,** 835–839
8. Carlberg, C. (1995) *Eur. J. Biochem.* **231,** 517–525
9. Haussler, M. R., Jurutka, P. W., Hsieh, J.-C., Thompson, P. D., Haussler, C. A., Selznick, S. H., Remus, L. S., and Whitfield, K. (1997) in *Vitamin D* (Feldman, D., Glorieux, F. H., and Pike, J. W., eds) pp. 149–177, Academic Press, San Diego
10. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) *Curr. Opin. Genet. & Dev.* **9,** 140–147
11. Freedman, L. P. (1999) *Cell* **97,** 5–8
12. Lanz, R. B., McKenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1999) *Cell* **97,** 17–27
13. St.-Arnaud, R., Messerlian, S., Moir, J. M., Omdahl, J. L., and Glorieux, F. H. (1997) *J. Bone Miner. Res.* **12,** 1552–1559
14. Takeyama, K.-I., Kitanaka, S., Takashi, S., Kobori, M., Yanagisawa, J., and Kato, S. (1997) *Science* **277,** 1827–1830
15. Reddy, G. S., and Tserng, K.-Y., (1989) *Biochemistry* **28,** 1763–1769
16. Omdahl, J. L., and May, B. K. (1997) in *Vitamin D* (Feldman, D., Glorieux, F. H., and Pike, J. W., eds) pp. 69–85, Academic Press, San Diego
17. Boyan, B. D., Dean, D. D., Sylvia, V. L., and Schwartz, Z. (1997) in *Vitamin D* (Feldman, D., Glorieux, F. H., and Pike, J. W., eds) pp. 259–273, Academic Press, San Diego
18. Kerry, D. M., Dwivedi, P. P., Hahn, C. N., Morris, H. M., Omdahl, J. L., and May, B. K. (1996) *J. Biol. Chem.* **271,** 29715–29721
19. Ohyama, Y., Ozono, K., Uchida, M., Yoshimura, M., Shinki, T., Suda, T., and Yamamoto, O. (1996) *J. Biol. Chem.* **271,** 30381–30385
20. Zierold, C., Darwish, H. M., and DeLuca, H. F. (1995) *J. Biol. Chem.* **270,** 1675–1678
21. Hahn, C. N., Kerry, D. M., Omdahl, J. L., and May, B. K. (1994) *Nucleic Acids Res.* **22,** 2410–2416
22. Liu, M., and Freedman, L. P. (1994) *Mol. Endocrinol.* **8,** 1593–1604
23. Koyama, H., Inaba, M., Nishizawa, Y., Ohno, S., and Morii, H. (1994) *J. Cell. Biochem.* **55,** 230–240

24. Dwivedi, P. P., Muscat, G. E. O., Bailey, P. J., Omdahl, J. L., and May, B. K. (1998) *J. Mol. Endocrinol.* **20,** 327–335
25. Thomas, R. S., Tymms, M. J., Seth, A., Shannon, M. F., and Kola, I. (1995) *Oncogene* **11,** 2135–2143
26. Yang, B. S., Hauser, C. A., Henkel, G., Colman, M. S., Beveren, C. V., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996) *Mol. Cell. Biol.* **16,** 538–547
27. Ho, I. C., Bhat, N. K., Gottschalk, L. R., Lindsten, T., Thompson, C. B., Papas, T. S., and Leiden, J. M. (1990) *Science* **250,** 814- 818
28. Andrew, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499
29. Ghysdael, J., Gegonne, A., Pognonec, P., Dernis, D., Leprince, D., and Stehelin, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1714–1718
30. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) *Eur. J. Biochem.* **211,** 7–18
31. Westermarck, J., Seth, A., and Kahari, V. M. (1997) *Oncogene* **14,** 2651–2660
32. Koizumi, S., Fisher, R. J., Fujiwara, S., Jorcyk, C., Bhat, N. K., and Papas, T. S. (1990) *Oncogene* **5,** 675–681
33. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) *Trends Biochem. Sci.* **23,** 213–216
34. Chen, K. S., and DeLuca, H. F. (1995) *Biochim. Biophys. Acta* **1263,** 1–9
35. Mitsunobu, H., Akasaka, K., Akinaga, S., Okabe, M., Nakano, H., Gomez, R., Wood, D., Uh, M., and Tamanoi, F. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2281–2285
36. Mandla, S., Boneh, A., and Tenenhouse, H. S. (1990) *Endocrinology* **127,** 2639–2647
37. Chen, M. L., Boltz, M. A., and Armbrecht, H. J. (1993) *Endocrinology* **132,** 1782–1788
38. Angel, P., and Karin, M. (1991) *Biochim. Biophys. Acta* **1072,** 129–157
39. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
40. Wasylyk, B., Wasylyk, C., Flores, P., Bergue, A., Leprince, D., and Stehelin, D. (1990) *Nature* **346,** 191–193
41. Ishiguro, H, Yamada, K, Ichino, N., and Nagatsu, T. (1998) *J. Biol. Chem.* **273,** 21941–21949
42. Tymms, M. J., Ng, A. Y. N., Thomas, R. S., Schutte, B. C., Zhou, J., Eyre, H. J., Sutherland, G. R., Seth, A., Rosenberg, M., Papas, T., Debouck, C., and Kola, I. (1997) *Oncogene* **15,** 2449–2462
43. Schwachtgen, J. L., Janel, N., Barek, L., Duterque-Coquillaud, M., Ghysdael, J., Meyer, D., and Kerbiriou-Nabias, D. (1997) *Oncogene* **15,** 3091–3102
44. Crepieux, P., Coll, J., and Stehelin, D. (1994) *Crit. Rev. Oncog.* **5,** 615–638
45. McNagny, K. M., Sieweke, M. H., Doderlin, G., Graf, T., and Nerlov, C. (1998) *EMBO J.* **17,** 3669–3680
46. Bradford, A. P., Wasylyk, C., Wasylyk, B., and Gutierrez- Hartmann, A. (1997) *Mol. Cell. Biol.* **17,** 1065–1074
47. Halle, J. P., Haus-Seuffert, P., Woltering, C., Stelzer, G., and Meisterernst, M. (1997) *Mol. Cell. Biol.* **17,** 4220–4229
48. Thomas, R. S., Tymms, M. J., McKinaly, L. H., Shannon, M. F., Seth, A., and Kola, I. (1997) *Oncogene* **14,** 2845–2855
49. Dittmer, J., Pise-Masison, C. A., Clemens, K. E., Choi, K.-S., and Brady, J. N. (1997) *J. Biol. Chem.* **272,** 4953–4958
50. Logan, S. K., Garabedian, M. J., Campbell, C. E., and Werb, Z. (1996) *J. Biol. Chem.* **271,** 774–782
51. Wotton, D., Ghysdael, J., Wang, S., Speck, N. A., and Owen, M. J. (1994) *Mol. Cell. Biol.* **14,** 840–850
52. Gegonne, A., Bosselut, R., Bailly, R. A., and Ghysdael, J. (1993) *EMBO J.* **12,** 1169–1178
53. Yang, C., Shapiro, L. H., Rivera, M., Kumar, A., and Brindle, P. K. (1998) *Mol. Cell. Biol.* **18,** 2218–2229
54. Jonsen, M. D., Peterson, J. M., Xu, Q. P., and Graves, B. J. (1996) *Mol. Cell. Biol.* **16,** 2065–2073
55. Graves, B. J (1998) *Science* **279,** 1000–1002
56. Peterson, J. M., Skalicky, Donaldson, L. W. J. J., McIntosh, L. P., Alber, T., and Graves, B. J. (1996) *Science* **269,** 1866–1869
57. Solomon, C., Sebag, M., White, J. H., Rhim, J., and Kremer, R.(1998) *J. Biol. Chem.* **273,** 17573–17578
58. Kola, I., Brookes, S., Green, A. R., Garber, R., Tymms, M., Papas, T. S., and Seth, A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7588- 759254
59. Slater, S. J., Kelly, M. B., Taddeo, F. J., Larkin, J. D., Yeager, M. D., McLane, J. A., Ho, C., and Stubbs, C. D. (1995) *J. Biol. Chem.* **270,** 6639–6643
60. Berry, D. M., Antochi, R., Bhatia, M., and Meckling-Gill, K. A. (1996) *J. Biol. Chem.* **271,** 16090–16096
61. Norman, A. W. (1997) in *Vitamin D* (Feldman, D., Glorieux, F. H., and Pike, J. W., eds) pp. 233–256, Academic Press, San Diego
62. Nemere, I., Schwartz, Z., Pedrozo, H., Sylvia, V. L., Dean, D. D., and Boyan, B. D. (1998) *J. Bone Miner. Res.* **13,** 1353–1359

Regulation of Rat Cytochrome P450C24 (*CYP24*) Gene Expression: EVIDENCE FOR FUNCTIONAL COOPERATION OF Ras-ACTIVATED Ets TRANSCRIPTION FACTORS WITH THE VITAMIN D RECEPTOR IN 1,25-DIHYDROXYVITAMIN D3-MEDIATED INDUCTION

Prem P. Dwivedi, John L. Omdahl, Ismail Kola, David A. Hume and Brian K. May

J. Biol. Chem. 2000, 275:47-55.
doi: 10.1074/jbc.275.1.47